COL4A1 Is Associated With Arterial Stiffness by Genome-Wide Association Scan

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Background—Pulse wave velocity (PWV), a noninvasive index of central arterial stiffness, is a potent predictor of cardiovascular mortality and morbidity. Heritability and linkage studies have pointed toward a genetic component affecting PWV. We conducted a genome-wide association study to identify single-nucleotide polymorphisms (SNPs) associated with PWV.

Methods and Results—The study cohort included participants from the SardiNIA study for whom PWV measures were available. Genotyping was performed in 4221 individuals, using either the Affymetrix 500K or the Affymetrix 10K mapping array sets (with imputation of the missing genotypes). Associations with PWV were evaluated using an additive genetic model that included age, age^2, and sex as covariates. The findings were tested for replication in an independent internal Sardinian cohort of 1828 individuals, using a custom chip designed to include the top 43 nonredundant SNPs associated with PWV. Of the loci that were tested for association with PWV, the nonsynonymous SNP rs3742207 in the *COL4A1* gene on chromosome 13 and SNP rs1495448 in the *MAGI1* gene on chromosome 3 were successfully replicated ($P=7.08\times10^{-7}$ and $P=1.06\times10^{-5}$, respectively, for the combined analyses). The association between rs3742207 and PWV was also successfully replicated ($P=0.02$) in an independent population, the Old-Order Amish, leading to an overall $P=5.16\times10^{-8}$.

Conclusions—A genome-wide association study identified a SNP in the *COL4A1* gene that was significantly associated with PWV in 2 populations. Collagen type 4 is the major structural component of basement membranes, suggesting that previously unrecognized cell-matrix interactions may exert an important role in regulating arterial stiffness. **(*Circ Cardiovasc Genet*. 2009;2:151-158.)**

Key Words: arterial stiffness ■ pulse wave velocity ■ genome-wide association scan ■ *COL4A1*, *MAGI1* ■ nonsynonymous SNP

Central arterial stiffening is one of the hallmarks of arterial aging. Carotid-femoral pulse wave velocity (PWV) is the preferred noninvasive measure of central arterial stiffness.[1] PWV is increased in patients with cardiovascular conditions such as hypertension, diabetes, metabolic syndrome, and atherosclerosis.[2] Furthermore, PWV is an independent predictor of hypertension[3] and of coronary heart disease and stroke in healthy subjects[4] and an independent predictor of mortality in the general population,[5] in hypertensive subjects,[6] in older community dwelling individuals,[7] and in patients with end-stage renal disease.[8] Thus, understanding the mechanisms of central arterial stiffening may lead to

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effective interventions that could improve PWV and favorably impact cardiovascular morbidity and mortality.

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Increasing central arterial stiffness has traditionally been thought to result from the breakdown of elastin in central arterial walls, due to the repeated cycles of distension and recoil of the central aorta, and from the deposition and cross-linking of collagen. There is an increasing recognition that arterial stiffening is influenced by lifestyle (eg, dietary salt consumption, and exercise) and is regulated by several signaling pathways (eg, nitric oxide).[2] Furthermore, gene expression studies,[9] heritability,[10,11] and linkage[11] analyses, and a genome-wide association study (GWAS) performed in 644 subjects from the Framingham Heart Study[12] are all consistent with the likely involvement of genetic factors in modulating the variability in PWV.

To look for these factors, we performed a GWAS in a large founder population (see online-only Data Supplement) from Sardinia. Furthermore, to confirm the validity of our findings we tested the association of the detected loci with PWV both in a second Sardinian cohort and in a separate founder population, the Old-Older Amish.

Methods

Study Sample

The SardiNIA study recruited 6148 men and women aged 14 to 102 years (62.5% of the eligible population)[10] from a cluster of 4 towns in the Lanusei valley on the island of Sardinia. The cohort includes 34 469 relative pairs, 4933 sibling pairs, 180 half-sibling pairs, 4014 first cousins, 4256 parent–child pairs, 675 grandparent–grandchild pairs, and 6400 avuncular pairs in addition to other more distant relatives. Most subjects (95%) had all 4 grand-parents born in Sardinia,[10] and environmental factors have remained relatively homogeneous. To achieve the accrual goal for the study, the project was advertised through provincial, religious, and municipal authorities; in local television, newspaper, and radio messages; through local physicians and by mailings and phone calls. All subjects underwent extensive phenotyping, which included assessment of traditional cardiovascular risk factors (eg, blood pressure and cholesterol fractions) with standard methodologies, as well as the assessment of arterial stiffness by PWV. Blood draws yielded lymphocytes for subsequent DNA extraction. All subjects provided a written informed consent for participation in the study that was approved by both the Sardinian and the National Institute on Aging's Institutional Review Boards.

PWV Measurement

PWV was measured in triplicate using nondirectional transcutaneous Doppler probes (model 810A, 9- to 10-Mhz probes, Parks Medical Electronics Inc, Aloha, Ore), as previously described[13]: A minimum of 10 arterial flow waves from the right common carotid artery and the right femoral artery were simultaneously recorded and averaged. PWV was calculated as distance divided by time. The distance traveled by the flow wave was measured with an external tape measure over the body surface and was calculated as the distance between the manubrium and the femoral sampling site, minus the distance between the manubrium and the carotid sampling site, and the time traveled by the flow wave was measured as the time delay between the feet of simultaneously recorded carotid and femoral arterial waveforms. The waveforms were simultaneously collected by 2 sonographers, 1 recording at the carotid site and 1 recording at the femoral site. All data were subsequently analyzed by a single investigator (A.S.) who was blinded to the clinical characteristics of the subjects. Details of a reproducibility study for PWV are provided in the Data Supplement. Forty-four individuals did not contribute PWV data to the analysis because of poor quality waveforms (n=21) or because of atrial fibrillation (n=23).

Genotyping

We took advantage of the relatedness among individuals in our sample to substantially reduce study costs.[14] Specifically, because our sample includes many large families, we reasoned that genotyping a relatively small number of markers in all individuals would allow us to identify shared haplotype stretches within each family. We could then genotype a subset of the individuals in each family at higher density to characterize the haplotypes in each stretch and impute missing genotypes in other individuals in the family.[14]

Genotyping was performed with the Affymetrix 10K and 500K Mapping Arrays in 3329 and 1412 individuals, respectively (436 subjects were genotyped with both chips). Individuals typed with the 500K array were specifically selected because they represented the largest families in our sample, not based on their phenotype. Furthermore, for the larger sibships in our cohort, both parents and 1 child were selected, whereas in the smaller sibships, only the 2 parents were selected. The lower density arrays were used to genotype everyone else. Except when parents and offsprings were genotyped in the same family, we tried to ensure that individuals genotyped with the high-density array were only distantly related to one another. In the 2893 individuals typed with only the 10K panel, we took advantage of the overlapping dataset and of the relatedness of the population to estimate the missing genotypes based on stretches of shared haplotypes, using a modified Lander-Green algorithm.[15,16] This approach for estimating missing genotypes is implemented in MERLIN (http://www.sph.umich.edu/csg/abecasis/MERLIN/) and is described in detail elsewhere[16,17] and in the Data Supplement.

Prior the imputation process, low-quality markers that could affect the accuracy of dosage estimates were removed. In particular, markers that met any of the following criteria were discarded: call rate ≤90%, minor allele frequency ≤5%, excess of Mendelian inconsistencies or departure from Hardy-Weinberg equilibrium. From the 10K and the 500K chips, we were able to analyze 7407 and 356 359 markers, respectively, resulting in a total of 362 129 markers after accounting for overlapping polymorphisms.[18] The genotype completeness rates exceeded 98%.

After completing this initial phase, we devised a custom chip to test for internal replication of our major findings and to eliminate possible genotyping errors. This chip consisted of 11 617 single-nucleotide polymorphisms (SNPs) and included, for each of the quantitative traits in the SardiNIA study,[10] the top SNPs that were associated with these traits in GWAS. Forty-three unique SNPs associated with PWV were included on this chip. This custom chip was used to type the remaining 1857 subjects recruited into the SardiNIA study who had not been typed with the 500K or 10K chips (denoted as SardiNIA stage 2). These individuals were not related to the individuals typed with the 500K chip (kinship coefficient=0) (see Data Supplement), and thus were a suitable cohort to serve as an internal replication sample. We considered a SNP to be internally replicated if the direction of effect was in the same direction as the initial study with $P<0.05$.

Statistical Analyses

To ensure adequate control of type I error rates, an inverse normal transformation was applied to PWV before analysis, to reduce the impact of outliers and minimize deviations from normality (supplementary Figure 1). This transformation involves ranking all available PWV values, transforming these ranks into quantiles and finally converting the resulting quantiles into normal deviates. To perform the genome-wide association analysis, a simple regression model was fitted and a variance component approach that modeled background polygenic effects was used to account for correlation between different observed phenotypes within each family.[17] The association analysis was performed using an additive genetic model and was adjusted for age, age^2, and sex. An initial Q/Q plot suggested that the genomic control parameter was inflated ($\lambda=1.14$), likely reflecting residual relatedness in this founder population. Therefore, the prob-

Table 1. Demographic and Clinical Characteristics of the SardiNIA Cohort (Overall and Stratified According to the Phase of Genotyping) and of the Old-Order Amish Study Cohort

	SardiNIA			
	Overall (n=6049)	Initial GWAS (n=4221)	Stage 2 (n=1828)	Old-Order Amish (n=826)
Age, y	43.7±17.6	43.1±17.4	44.1±16.9	46.4±15.1
Sex (male)	2569 (42.5)	1841 (43.6)	728 (39.8)	443 (53.6)
Smoking (ever)	1237 (20.3)	850 (20.1)	387 (21.2)	211 (25.5)
Height, cm	159.9±9.1	159.9±9.0	160.1±9.1	167.1±8.8
Weight, kg	64.9±13.3	64.8±13.3	65.0±13.2	73.8±12.7
BMI, kg/m^2	25.3±4.7	25.3±4.7	25.3±4.6	26.4±4.3
Systolic blood pressure, mm Hg	125±18	125±18	125±18	120±15
Diastolic blood pressure, mm Hg	77±11	77±11	77±11	75±9
Heart rate, beats/min	67±11	67±11	68±11	64±9
PWV, cm/s	670±217	669±209	672±207	547±143
Total cholesterol, mg/dL	209±43	208±42	210±42	210±47
Triglycerides, mg/dL	88±68	87±68	89±69	72±44
LDL, mg/dL	127±36	127±35	127±36	139±42
HDL, mg/dL	64±15	64±15	64±15	56±15
Fasting glucose, mg/dL	90±23	90±23	90±25	87±10
Creatinine, mg/dL	0.8±0.2	0.8±0.2	0.8±0.2	0.8±0.2
Antihypertensive medications	752 (12.4)	544 (12.2)	208 (11.4)	18 (2.2)

Data are expressed as mean±SD or n (%). BMI indicates body mass index; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

ability values were adjusted according to the genomic control method[19] (supplementary Figure 2). The probability values derived from the initial GWAS and from the association tests on the individuals genotyped with the custom chip were then combined using the *z*-scores meta-analysis methods, and taking into account the number of subjects analyzed in each set and the direction and magnitude of the estimated effect.

External Replication

The top findings from our study were tested in 813 subjects from a genetically distant second-founder population, the Old-Order Amish of Lancaster, Pa. These included healthy Amish subjects enrolled in 2 studies, the Heredity and Phenotype Intervention (HAPI) Heart Study and the Amish Longevity Study (ALS). The HAPI Heart Study was initiated in 2002 to measure the cardiovascular response to 4 short-term interventions affecting cardiovascular risk factors and to identify the genetic and environmental determinants of these responses.[20] The ALS was initiated in 2000 to identify the genetic factors associated with living to an old age, and recruited Amish individuals living to age 92 years or older, their offspring, and the offspring spouses.[21] PWV was assessed with the CompliorSP device (Artech Medical, Pantin, France) before the interventions were administered, and genotyping was performed with an Affymetrix 500K chip. GWAS in the Old-Order Amish were adjusted for family structure, and the data transformation and statistical analyses were performed in an analogous manner to those in the SardiNIA study.

Results

After excluding subjects with atrial fibrillation or poor quality PWV tracings, a total of 4221 and 1828 Sardinians were considered for the initial GWA analysis and for the internal replication (SardiNIA stage 2), respectively. The demographic and clinical characteristics of the study cohort are shown in Table 1. The mean age was 43.7±17.6 years (range, 14 to 102 years), 58% were women, 20% reported a history of smoking, 29% were hypertensive, 5% were diabetics, and only 1% reported a clinical history of myocardial infarction. As expected,[2] PWV increased with advancing age in a quadratic fashion (Figure 1).

We first conducted GWAS to survey the genome for common variants associated with PWV. Figure 2 graphically summarizes the associations of PWV with the >329 129 SNPs with a minor allele frequency >5% that passed quality control checks and transmission disequilibrium testing. The top 100 hits are shown in supplementary Table 1. Promising findings were noted on chromosomes 1, 2, 4, 12 to 14, 17, 20, and 21, encompassing 18 SNPs with $P<2\times10^{-5}$.

To further evaluate these initial results, a secondary custom chip from Affymetrix was devised based on the top findings from the GWAS. For PWV, the top 85 SNPs were considered; of these, 43 were not in strong linkage disequilibrium and were included in the custom chip. The 1828 individuals typed with this custom chip were genetically independent from those genotyped in the initial analysis. Thus, the custom chip helped validate the initial findings, and provided an internal replication within the Sardinian cohort. Of the 43 SNPs that were included in the custom designed chip, 2 SNPs, rs3742207 and rs1495448 in the *COL4A1* and *MAGI1* genes, respectively, were significantly associated with PWV with the same directionality of effect as in the GWAS (Table 2). Furthermore, when the results of the initial GWAS study group and the stage 2 study group were combined in a meta-analysis (Table 2), the association of the C allele of rs3742207 with increased PWV was strengthened ($P=7.08\times10^{-7}$), whereas the association of the T allele of rs1495448 with increased PWV was weakened ($P=1.07\times10^{-5}$).

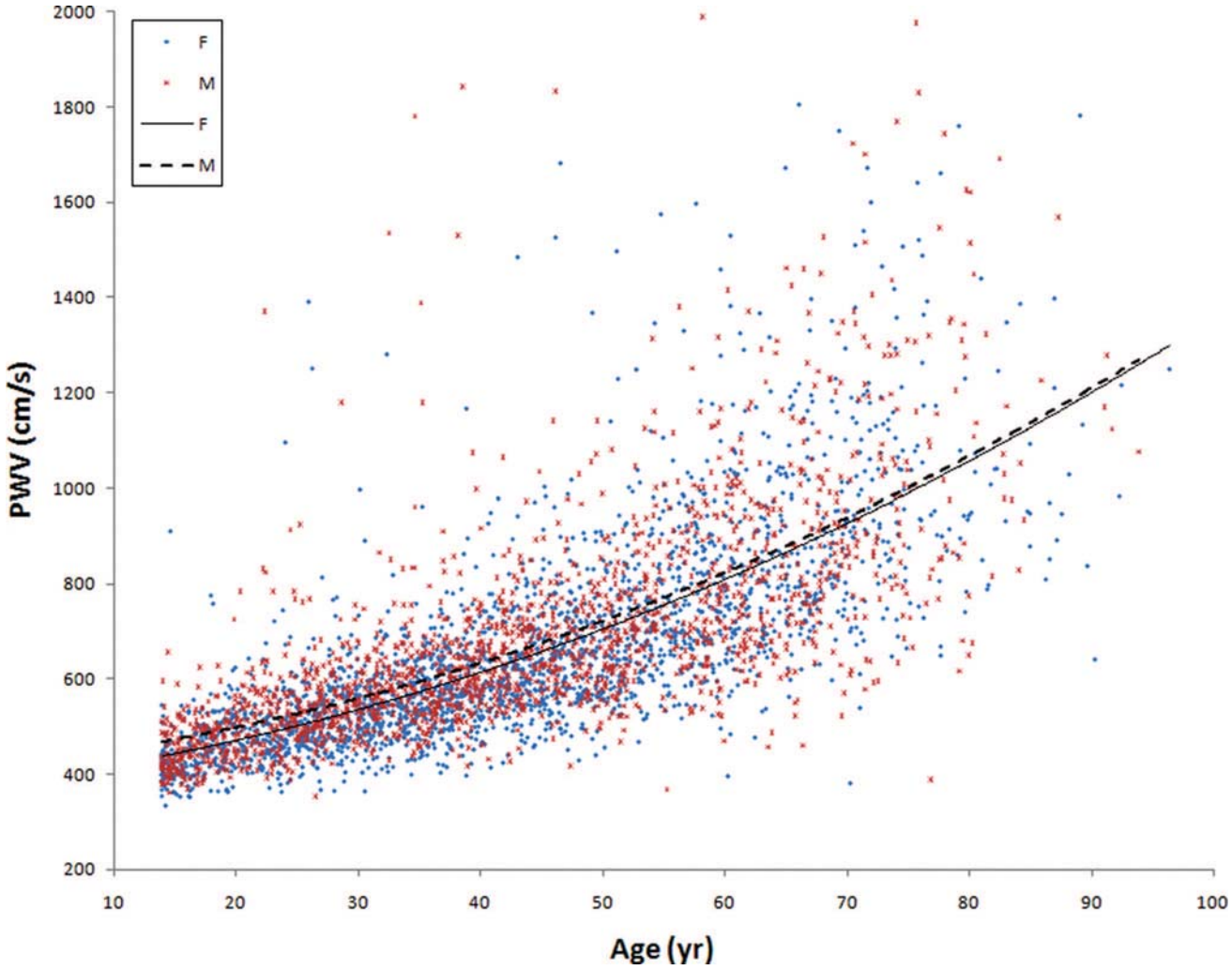

Figure 1. The relationship of PWV and age in men and women in the SardiNIA study cohort who were genotyped with the 500K/10K gene array set. The best fit regression curves (quadratic) are shown.

We repeated the association analyses using models that adjusted for mean arterial pressure, creatinine, and the use of blood pressure lowering medications, which are important covariates of arterial stiffness, in addition to age, age^2, and sex that were adjusted for in the base models. The association of rs3742207 with PWV was slightly strengthened, whereby the probability value decreased from 5.94×10^{-5} to 1.78×10^{-5}. Next, we excluded subjects on antihypertensive medications (n=544) and subjects on dialysis (n=10), and repeated the analyses adjusting for age, age^2, sex, mean arterial pressure, and creatinine. The probability value for the association of rs3742207 with PWV was 2.48×10^{-5}. In this last model, the probability value was 3.19×10^{-5} when diabetic individuals (n=50) were excluded. However, the genomic control parameters for these 3 additional models were higher than the one for the base model (λ=1.16, 1.17, and 1.17, respectively, compared with 1.14 of the initial model). Conversely, the association of PWV with rs1495448 (in the *MAGI1* gene) was

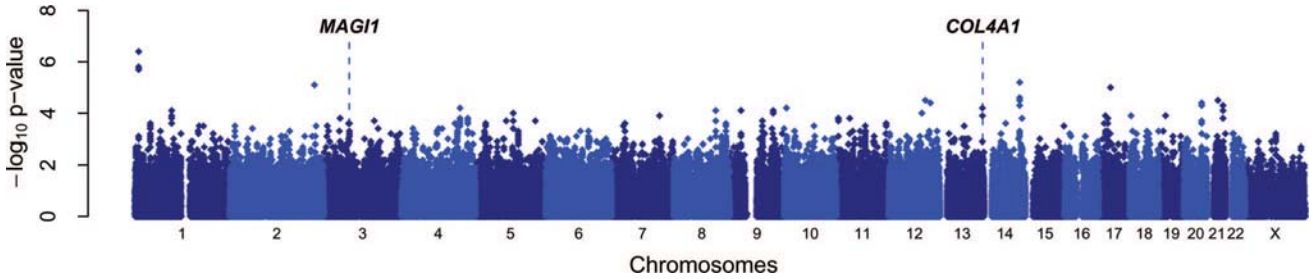

Figure 2. Summary of GWAS for PWV in the SardiNIA study cohort. The −log10 of the probability values for the associations of PWV with 362 169 SNPs that passed quality control filters are plotted according to the positions of these SNPs along the p to q arms of each chromosome (1 to 22, X). The positions of *COL4A1* and *MAGI1*, the 2 genes that were replicated in the SardiNIA stage 2 analysis, are highlighted.

Table 2. Summary of Association Results for rs3742207 and rs1495448

Study	N	Allele (+/−)	Frequency	Effect (SE)	*P*
COL4A1					
SardiNIA	4221	C/A	0.44	21.0 (4.7)	5.94×10^{-5}
SardiNIA stage 2	1828	C/A	0.42	15.2 (5.1)	0.0035
Combined SardiNIA					7.08×10^{-7}
Old-Order Amish	813	C/A	0.55	16.4 (8.2)	0.0218
Combined SardiNIA and Old Order Amish					5.16×10^{-8}
MAGI1					
SardiNIA	4221	T/G	0.42	8.5 (4.5)	2.76×10^{-4}
SardiNIA stage 2	1828	T/G	0.46	11.0 (5.0)	0.013
Combined SardiNIA					1.07×10^{-5}
Old-Order Amish	813	T/G	0.50	2.3 (8.7)	0.49
Combined SardiNIA and Old Order Amish					1.23×10^{-5}

This table summarizes the association results with PWV for the top SNPs, rs3742207 (*COL4A1*) and rs1495448 (*MAGI1*). Alleles refer to the forward strand and are ordered such that the first allele (+) is associated with increased PWV values. Effect sizes and SEs (expressed in cm/s) are derived from models that analyzed non-normalized PWV, whereas *P* values are calculated from models that analyzed the normalized trait as described in the text.

weakened when the analyses were repeated using these 3 sets of additional adjustments ($P=0.0014$, $P=0.0038$, and $P=0.0081$, respectively).

The associations of the 2 loci rs3742207 and rs1495448 with PWV were further evaluated in the Amish population, a genetically distant founder population of European ancestry. The HAPI Heart Study and the ALS participants underwent both assessment of PWV and genotyping using the Affymetrix 500K chip. We confirmed that allele C of the SNP rs3742207 (the more frequent allele in the Amish population) was associated with PWV ($P=0.02$) with a comparable effect size (Table 2), whereas rs1495448 was not ($P=0.49$; Table 2). Combining SardiNIA, SardiNIA stage 2, and the Old-Order Amish yielded an overall $P=5.16\times10^{-8}$ for the association between rs3742207 and PWV.

The heritability of PWV in the SardiNIA study is 0.226 (adjusted for age, sex, and age-by-sex interaction).[10] The proportion of variance in PWV (which is equivalent to the proportion of the heritable fraction) that is explained by rs3742207 is 0.87%. The overall effect size is 18.9 cm/s (calculated as a weighted average of the effect observed in each of the 3 study cohorts, in which the weights correspond to those used in the meta-analysis).

The replicated SNP rs3742207 is a common nonsynonymous coding polymorphism located in exon 45 of the *Col4A1* gene. This polymorphism involves a substitution of adenine by cytosine resulting in an amino acid change from glycine to histidine at position 1334, which is located in a central region of the protein that consists of multiple triple-helix repeat domains. Nonetheless, further studies are necessary to determine whether the true causal variant is this SNP or another one, which according to linkage disequilibrium structure (Figure 3),[12,22,23] seems to lie in exon 45 or nearby.

Discussion

We conducted a GWAS in the SardiNIA cohort, and found that SNP rs3742207 in the *COL4A1* gene was significantly associated with PWV. Furthermore, this locus was successfully replicated both in an independent sample within the SardiNIA cohort, and in the Old-Order Amish population, an external genetically distant founder population of European ancestry, suggesting that this *COL4A1* variant may have an effect in other populations.

Collagen Type 4

There are 6 different types of type 4 collagen-α chains (α1 to α6). Each one is encoded by a different gene and comprises repeating triple-helical domains with a characteristic G-X-Y motif interposed between the amino terminus and a globular C-terminus. These α chains assemble into triple helices to form type 4 collagen. The collagen type 4 α1 molecule consists of 20 triple helical repeats G-X-Y(X), where the first position is always a Glycine residue. Mutations in this Glycine residue cause Mendelian disorders characterized by small vessel,[24] or small and large vessels[25] angiopathy.

Type 4 collagen had not previously been considered to be involved in regulating arterial stiffness. Unlike collagen types 1 and 3, which are constituents of the extracellular matrix that are found in the medial layer of the arterial walls where they impart the tensile strength to the arteries, type 4 collagen is a structural component of basement membranes. At present, there is no functional evidence implicating *COL4A1* as a determinant of PWV. Nonetheless, a speculative discussion of putative mechanisms through which *COL4A1* may influence arterial stiffness is provided in the Data Supplement.

Studies of the Genetics of Arterial Stiffness

Heritability studies have consistently concluded that a genetic component likely underlies the variance in arterial stiff-

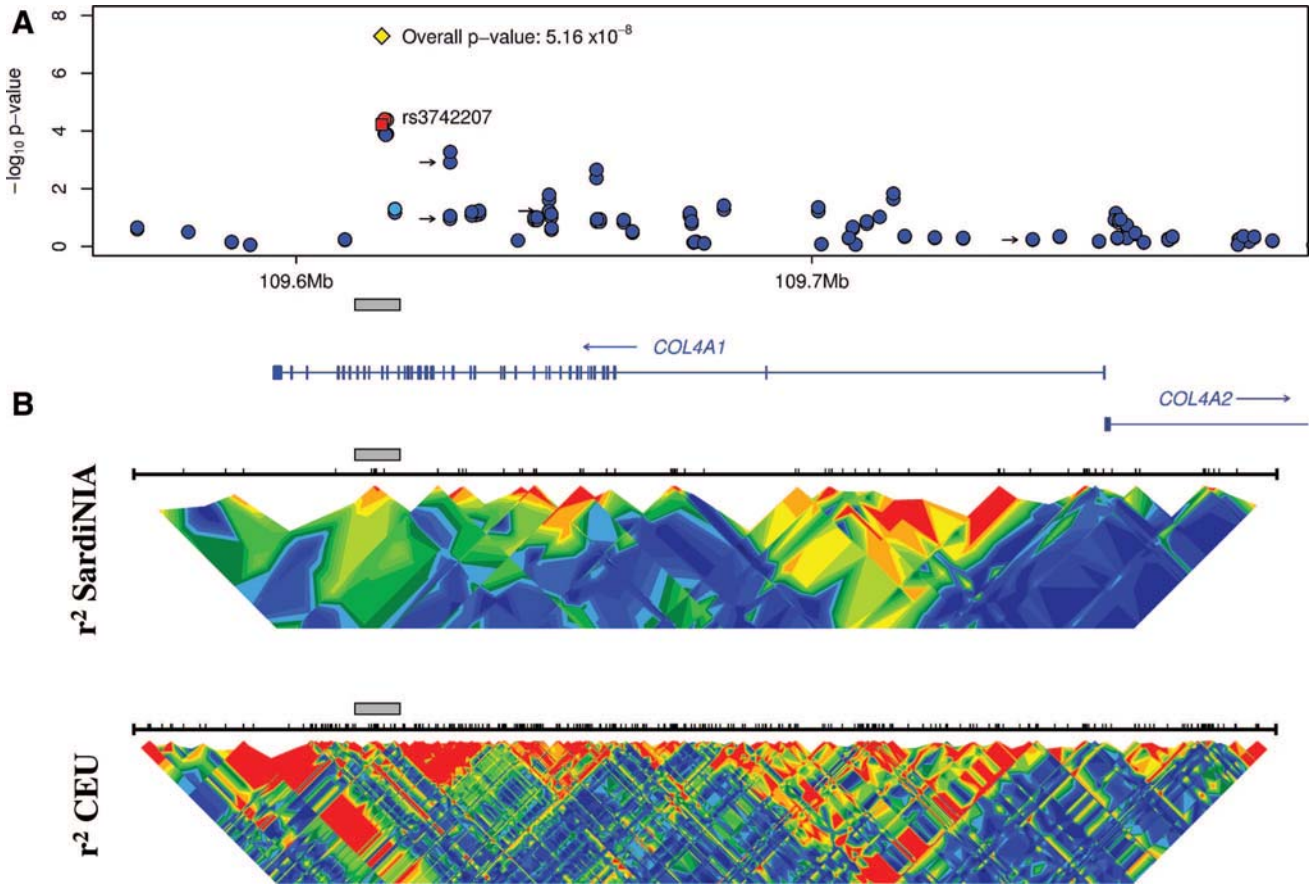

Figure 3. A, Summary of the association with PWV in the *COL4A1* region in the SardiNIA study. Dots represent all the markers in this region that were analyzed, colored according to their LD (r^2) with rs3742207, which is represented by a red square. LD coloring ranges from red (high LD), to green (moderate LD), to blue (low LD). The yellow diamond indicates the overall probability value for rs3742207 obtained from combining the results of SardiNIA, SardiNIA stage 2, and the HAPI Heart Study. Black arrows denote markers that were also analyzed by the Framingham Heart Study,[12] and they are from left to right: rs2131939, rs10492497, rs496916, and rs2391823. B, Summary of the patterns of disequilibrium in the SardiNIA study cohort and in the CEU (Utah residents with ancestry from Northern and Western Europe) HapMap population,[22] where r^2 values are calculated as described[23] and colored as in panel A. The gray bar marks the region of association and facilitates comparisons among the panels.

ness.[26–28] Similarly, results of linkage studies for noninvasive indices of arterial stiffness[11,27,28] suggested an underlying genetic component, even though the findings among these studies did not necessarily overlap.

Previous association studies that examined the genetic underpinnings of arterial stiffness focused mostly on polymorphisms in candidate genes that are believed to be involved in regulating arterial structure and/or function, such as nitric oxide synthase,[29] angiotensin II type 1 receptor,[30] collagen 1,[26] G-protein β-3 subunit,[31] β-adrenergic receptors,[32] fibrillin 1,[33] and C-reactive protein.[34] These candidate gene studies, which were conducted in single populations, did not attempt replication in other populations, and often yielded discrepant results.[27,29]

The first GWAS evaluating SNPs associated with arterial stiffness was performed in ≈644 participants in the Framingham Heart Study, using a 100K Affymetrix GeneChip array.[11] None of the associations with the various markers of arterial stiffness reached genome-wide significance in that study. The 100K chip that was used did not include rs3742207 or any neighboring SNP in the same linkage disequilibrium (LD) block (supplementary Figure 3). It did include 7 other SNPs in the *COL4A1* gene region, 3 of which were associated ($0.01<P<0.05$) with PWV in age-adjusted and sex-adjusted analyses (available at http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=search&db=gap&term=carotid-femoral%20pulse%20wave%20velocity&doptcmdl=SAnalyses). Of note, some of these markers were also associated with PWV in SardiNIA (Figure 3A) but with higher probability values than SNP rs3742207.

The GWAS in the SardiNIA study was performed in a larger cohort and with a much denser SNP map, and showed, for the first time, a link between a polymorphism in the collagen type 4 α1 gene and arterial stiffness. Each copy of the minor allele (C) of rs3742207 is associated with a 21 cm/s higher PWV, such that homozygotes for this minor allele have an ≈42 cm/s (6.3%) higher PWV than homozygotes for the major allele, an effect size comparable with those reported in GWAS of other traits, including ones recently studied in the Sardinian cohort.[18,35,36] Importantly, in a cohort of 1678 Danes aged 40 to 70 years, a PWV increase of 34 cm/s was associated with a 2.9% increase in age-adjusted and sex-adjusted cardiovascular mortality.[5] Interestingly, rs3742207 was recently found to be associated with the prevalence of myocardial infarction in Japanese individuals.[37]

Limitations

This study was conducted primarily in a founder population in SardiNIA, so caution should be exercised in extending these results to other populations. However, the values and distributions of PWV in our study did not significantly differ from those obtained in other out-bred populations. Furthermore, and in contrast to the microisolates where the gene pool is restricted to a few variants, the number of founders of the current day Sardinian population is large enough to encompass most of the existing alleles in the European population, although with some differences in frequency. Importantly, recent studies from the SardiNIA project[18,35,36] have demonstrated that findings in SardiNIA are reproducible in other populations; and specifically in this study, we were able to replicate the association of our top finding in an independent and genetically distant founder population of European ancestry. Nonetheless, additional studies in populations of different ethnic origin are needed to further replicate and extend our finding.

Conclusions

Using GWAS, we found that a SNP in the *COL4A1* gene is strongly associated with PWV, an established independent predictor of adverse cardiovascular outcomes. Collagen type 4 is the major structural component of basement membranes, suggesting that previously unrecognized cell-matrix interactions may exert an important role in regulating arterial stiffness. Further work is needed to elucidate these mechanisms, and this could potentially lead to the development of novel interventions aimed at delaying or preventing the risks associated with accelerated arterial stiffening. This would help fulfill, in part, the high expectations for breakthroughs in basic science and in clinical medicine that are engendered by modern-era genetics.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Pulse wave velocity, a noninvasive index of central arterial stiffness, is a potent predictor of cardiovascular mortality and morbidity. Heritability and linkage studies have pointed toward a genetic component affecting pulse wave velocity. We conducted a genome-wide association study to identify single-nucleotide polymorphisms associated with pulse wave velocity. Analyses were performed in 4221 participants in the SardiNIA study, a founder population. We found that the nonsynonymous single-nucleotide polymorphisms rs3742207 in the *COL4A1* gene was significantly associated with pulse wave velocity. This locus was successfully replicated both in an independent sample within the SardiNIA cohort and in the Old-Order Amish population, an external genetically distant founder population of European ancestry, with an overall $P=5.16\times10^{-8}$. Collagen type 4 is the major structural component of basement membranes, suggesting that previously unrecognized cell-matrix interactions may exert an important role in regulating arterial stiffness, which is an established independent predictor of adverse cardiovascular outcomes. Further work is needed to elucidate these mechanisms, but this could potentially lead to the development of novel interventions aimed at delaying or preventing the risks associated with accelerated arterial stiffening.

SUPPLEMENTAL MATERIAL

for the manuscript

***COL4A1* Is Associated With Arterial Stiffness By Genome Wide Association Scan.**

Tarasov KV et al.

Introduction

Founder population

In population genetics, founder effect refers to the establishment of a new colony by a very small number of individuals from a larger population, often many hundreds or thousands of years ago. When such founder groups expand to large modern populations without substantial in-migration, they facilitate detailed genealogical research and reconstruction of extended multigenerational pedigrees; and the relatedness and relative environmental homogeneity make it easier to determine the alleles that contribute to the values of a complex phenotype or to a disease. [1-3]

Methods

Reproducibility of PWV in SardiNIA

Each measurement of PWV was performed by 2 sonographers, with one recording the waveforms at the carotid site, and the other simultaneously recording the waveforms at the femoral site. Because several sonographers participated in the collection of the PWV data, a reproducibility study was performed on 3 subjects who underwent repeated measurements of

PWV using random combinations of testers (there were 5 testers at the carotid site and 3 testers at the femoral site). Each subject underwent approximately 45 measurements of PWV. The coefficient of variation for PWV ranged from 7.2 to 10.6%. Both general linear models as well as mixed effects models showed that only inter-subject differences significantly contributed to the overall error in the measurement of PWV, whereas carotid testers, femoral testers, and the interaction between carotid and femoral testers did not.

Imputations

For individuals who were genotyped with the 500k chip, the SNP being tested is coded as 0, 1, or 2, depending on the number of copies carried of an arbitrary reference allele. If this SNP was not included in the 10K chip, than for individuals with missing genotype data (i.e. those genotyped only with the 10k chip), the Lander-Green algorithm was used to estimate the number of copies of this allele that are being carried, which was assigned a score ranging between 0 and 2[4]. Importantly, this score represents a probabilistic estimate of the number of copies of the allele, and it incorporates information on allele frequency, the genotype of relatives for the SNP of interest, their degree of relatedness, and data on flanking markers. Thus, this score need not be an ordinal value of 0, 1 or 2, but can be a fractional number that ranges between 0 and 2, which enables uncertain genotypes to be accounted for. For computational efficiency, the Lander-Green algorithm was applied to sub-pedigrees, each including no more than 20-25 individuals, resulting in a dataset where the average analysis unit consisted of a family with 12.3 members and 3.2 generations.

Kinship coefficient:

Kinship coefficient in the SardiNIA study was determined on the basis of family structure, not genotype data. Family structure, in turn, was determined on the basis of self-reported information, which allowed us to construct family genealogy trees that were up to five generations deep. However, analytic constraints required us to ignore any relatedness that was not observed within these 5 generations. All the first and second degree relationships were verified based on the genotype data using GRR software[4], and adjusted if necessary.

Comparison of PWV protocols in SardiNIA and HAPPI Heart:

The device used to measure PWV in HAPI Heart was the Complior® SP device (Artech Medical, Pantin, France), which differs than the custom-designed device that was used in SardiNIA. However, both devices have been validated[5, 6], and PWV measured with either of these devices has been shown to be an independent predictor of mortality in large epidemiological studies[7, 8]. Importantly, since the raw PWV data from the 2 studies were not combined, rather the results were combined in the meta-analysis; and since the adjustment for family structure and the data transformation and the statistical analyses in HAPI Heart were performed in a manner that was analogous to the way they were performed in SardiNIA, we don't feel that the results of the genetic analyses could be an artifact of the minor methodological differences that arise from the use of two different devices. In fact, several meta-analyses have recently been performed for many other quantitative traits [3, 9-11], and the

combined results have consistently pointed to genes that were associated with the trait in most of the cohorts in spite of differences in the devices or methodologies used to perform the measurements.

Discussion

Collagen Type 4

Type 4 Collagen had not previously been considered to be involved in regulating arterial stiffness. Unlike Collagen types 1 and 3, which are constituents of the extracellular matrix that are found in the medial layer of the arterial walls where they impart the tensile strength to the arteries, type 4 Collagen is a structural component of basement membranes. Basement membranes surround vascular smooth muscle cells in the media. During the aging process within arteries, smooth muscle cells manufacture, secrete and activate type 2 matrix metalloprotease (MMPII), a collagenase whose substrate is type 4 Collagen. Degradation of type 4 Collagen in the basement membranes permits these cells to invade the internal elastic membrane and enter the subendothelial space. These cells then proliferate and secrete matrix, resulting in a diffusely thickened intima that interferes with endothelial function and also alters the transduction of mechanical forces imparted by flow and pressure of the blood[12]. The *COL4A1* polymorphism described in the present study could impact on age-associated arterial stiffening by affecting the binding of MMPII to collagen 4, its substrate. Alternatively, this polymorphism could affect the polymerization of the Collagen 4α1 molecules not only rendering basement membranes more susceptible to metalloprotease

digestion but also differentially affecting formation of the advanced glycation end-products which are known to influence the assembly of type 4 Collagen[13] and to modulate arterial stiffness.

Beyond its role as a structural component of the basement membrane, type 4 Collagen also provides structural support and anchorage for cells, serves as a ligand for cell surface receptors, modulates endothelial cell proliferation, and regulates angiogenesis and tumor growth. Thus, allelic variants of *COL4A1* could differentially affect the permeability of the basement membrane, or the cell-matrix and/or cell-basement membrane interactions of the endothelial or smooth muscle cells in the arterial wall. In this context, it is noteworthy that the *MAGI1* gene product also plays a role in cell-cell interactions, as a scaffolding protein at cell-cell junctions and as a mediator of vascular endothelial cell adhesions[14].

Finally, allelic variants could differentially influence the type or quantity of growth factors secreted by these cells (e.g. TGF-β), which, in turn, would influence signaling cascades e.g. SMAD signaling, initiated by TGF-β receptor activation, which leads to increased production of collagen I and III matrix proteins known to directly affect arterial stiffness.

We should note that at the present time there is no functional evidence implicating *COL4A1* as a determinant of PWV. Therefore the foregoing discussion of its putative role in influencing arterial stiffness remains speculative. Furthermore, by using standard prediction algorithms we could not find any evidence that the Glycine to Histidine substitution at position 1334 that is associated with the rs3742207 SNP leads to any significant changes in the 3-dimensional views of protein structure or domain interactions, even though this replacement could lead to changes in the local charge.

Supplementary Table S1.

The top 100 SNPs associated with PWV in the initial GWAS in the SardiNIA cohort.

SNP	CHR	POSITION	MARKER	ALLELE1	ALLELE2	FREQ1	EFFECT	SE	H2	LOD	PVALUE	gc_p-value*	GENE
SNP_A-1945628	1	11063525	rs1194820	G	T	0.771	-0.108	0.021	1.151	5.638	3.48E-07	2.33E-06	SRM, U88966, FRAP1, EXOSC10
SNP_A-2146504	1	11139324	rs1057079	T	C	0.764	-0.103	0.021	1.07	5.172	1.06E-06	6.09E-06	SRM, U88966, FRAP1, EXOSC10
SNP_A-2248327	1	11064909	rs910660	G	A	0.771	-0.102	0.022	1.025	4.898	2.04E-06	1.07E-05	SRM, U88966, FRAP1, EXOSC10
SNP_A-1958507	20	46472188	rs732791	A	T	0.763	0.099	0.021	0.983	4.82	2.46E-06	1.26E-05	-
SNP_A-4226147	1	95627748	rs12032935	G	T	0.621	-0.084	0.018	0.933	4.722	3.11E-06	1.55E-05	-
SNP_A-1810120	13	109616599	rs3742207	T	G	0.559	-0.085	0.018	0.994	4.682	3.43E-06	1.68E-05	COL4A1
SNP_A-4277712	4	151475987	rs10031796	G	A	0.91	0.141	0.03	0.9	4.638	3.81E-06	1.84E-05	DCAMKL2, AB209181
SNP_A-1905780	20	46485633	rs16993776	A	G	0.765	0.097	0.021	0.932	4.632	3.86E-06	1.86E-05	-
SNP_A-1871247	14	91106545	rs1704693	C	G	0.722	-0.089	0.019	0.888	4.6	4.18E-06	1.99E-05	C14orf161
SNP_A-1934075	1	95632284	rs12568065	C	T	0.619	-0.083	0.018	0.897	4.573	4.45E-06	2.11E-05	-
SNP_A-2138220	1	95627844	rs12040149	C	A	0.62	-0.083	0.018	0.896	4.57	4.49E-06	2.12E-05	-
SNP_A-4226109	18	4384577	rs7244876	A	G	0.933	-0.164	0.036	0.924	4.555	4.65E-06	2.19E-05	-
SNP_A-2019391	21	39497183	rs7281540	T	C	0.834	-0.107	0.023	0.883	4.546	4.76E-06	2.23E-05	AB080587, BRWD1, AJ238214, DSCR2
SNP_A-1811989	14	91101729	rs1743074	T	C	0.723	-0.089	0.019	0.875	4.543	4.79E-06	2.24E-05	C14orf161
SNP_A-2304434	17	29772173	rs756882	G	A	0.805	-0.102	0.022	0.906	4.538	4.85E-06	2.27E-05	-
SNP_A-4301686	1	95618898	rs4949963	T	A	0.619	-0.082	0.018	0.877	4.495	5.38E-06	2.48E-05	-
SNP_A-2282847	14	91089550	rs17799683	G	A	0.892	-0.124	0.028	0.824	4.413	6.55E-06	2.94E-05	C14orf161

SNP_A-2019381	21	39437639	rs4816613	A	G	0.82	-0.104	0.023	0.876	4.353	7.56E-06	3.33E-05	AB080587, BRWD1, AJ238214, DSCR2
SNP_A-2019382	21	39437774	rs4817994	C	G	0.82	-0.104	0.023	0.876	4.353	7.57E-06	3.33E-05	AB080587, BRWD1, AJ238214, DSCR2
SNP_A-2201277	13	109617587	rs589985	A	G	0.563	-0.082	0.018	0.915	4.337	7.85E-06	3.44E-05	COL4A1
SNP_A-4201480	5	97239524	rs11135532	C	G	0.84	-0.111	0.025	0.928	4.317	8.25E-06	3.59E-05	-
SNP_A-2016988	21	26186455	rs454017	C	A	0.928	0.148	0.033	0.812	4.303	8.53E-06	3.69E-05	APP
SNP_A-2301509	13	109617144	rs694225	G	A	0.563	-0.081	0.018	0.903	4.292	8.76E-06	3.78E-05	COL4A1
SNP_A-4247337	4	151475498	rs7659254	C	T	0.913	0.135	0.03	0.805	4.244	9.84E-06	4.18E-05	DCAMKL2, AB209181
SNP_A-1789811	13	109617363	rs1213026	G	A	0.562	-0.081	0.018	0.887	4.211	1.07E-05	4.47E-05	COL4A1
SNP_A-2019405	21	39588141	rs2836978	T	C	0.849	-0.109	0.025	0.838	4.207	1.08E-05	4.51E-05	AB080587, BRWD1, AJ238214, DSCR2
SNP_A-4233332	8	109653323	rs13257259	T	A	0.62	0.081	0.018	0.85	4.197	1.10E-05	4.61E-05	KIAA0103
SNP_A-1903882	20	46492084	rs993425	G	C	0.728	0.088	0.02	0.859	4.168	1.18E-05	4.89E-05	-
SNP_A-2166248	20	46471877	rs732790	T	A	0.782	0.095	0.022	0.851	4.16	1.20E-05	4.98E-05	-
SNP_A-2019388	21	39479848	rs2297255	G	C	0.818	-0.1	0.023	0.823	4.145	1.25E-05	5.14E-05	AB080587, BRWD1, AJ238214, DSCR2
SNP_A-1893621	1	177878471	rs10797677	A	G	0.638	-0.08	0.018	0.818	4.138	1.27E-05	5.21E-05	-
SNP_A-2117728	1	95602998	rs4950054	C	T	0.618	-0.079	0.018	0.808	4.111	1.36E-05	5.51E-05	-
SNP_A-4205602	4	151471517	rs1459751	T	G	0.91	0.132	0.03	0.795	4.098	1.40E-05	5.66E-05	DCAMKL2, AB209181
SNP_A-4284308	17	23127161	rs4796052	C	T	0.603	0.078	0.018	0.806	4.061	1.53E-05	6.12E-05	NOS2A
SNP_A-2019385	21	39452857	rs6517516	G	A	0.822	-0.1	0.023	0.814	4.057	1.54E-05	6.17E-05	AB080587, BRWD1, AJ238214, DSCR2
SNP_A-2080047	4	151470110	rs6535720	G	A	0.913	0.122	0.028	0.661	4.055	1.55E-05	6.20E-05	DCAMKL2, AB209181
SNP_A-1846050	17	23148826	rs16949	T	C	0.624	0.076	0.018	0.757	4.013	1.72E-05	6.77E-05	NOS2A
SNP_A-4248604	17	17194880	rs242247	G	A	0.935	0.156	0.036	0.821	3.988	1.83E-05	7.13E-05	NT5M
SNP_A-1997660	9	110671392	rs544828	G	A	0.788	0.09	0.021	0.745	3.979	1.86E-05	7.26E-05	-
SNP_A-2099062	14	96989660	rs1994179	G	C	0.629	0.075	0.018	0.73	3.975	1.88E-05	7.33E-05	-
SNP_A-2156877	9	110673454	rs498102	A	G	0.787	0.089	0.021	0.734	3.927	2.12E-05	8.10E-05	-
SNP_A-	11	74919329	rs661928	T	C	0.696	-0.084	0.02	0.836	3.924	2.13E-	8.15E	AL833941

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SNP_A-1954971	10	123764372	rs7919547	G	A	0.582	-0.076	0.018	0.772	3.873	2.41E-05	9.07E-05	TACC2
SNP_A-4261235	6	114017197	rs4555958	G	A	0.669	-0.08	0.019	0.79	3.873	2.41E-05	9.07E-05	-
SNP_A-2148911	11	42404449	rs4755605	A	G	0.875	0.105	0.025	0.672	3.856	2.51E-05	9.40E-05	-
SNP_A-4288247	2	218565238	rs1424917	C	G	0.713	0.082	0.02	0.769	3.851	2.54E-05	9.50E-05	TNS1
SNP_A-1894443	10	123764416	rs7919851	A	G	0.582	-0.075	0.018	0.767	3.844	2.59E-05	9.64E-05	TACC2
SNP_A-4275345	4	151495409	rs4532220	C	A	0.909	0.127	0.03	0.744	3.839	2.62E-05	9.74E-05	DCAMKL2, AB209181
SNP_A-2126969	12	107402604	rs4388939	A	G	0.909	0.124	0.03	0.712	3.835	2.64E-05	9.82E-05	-
SNP_A-4288324	9	110673051	rs2767006	C	A	0.788	0.088	0.021	0.716	3.832	2.66E-05	9.88E-05	-
SNP_A-2175210	14	91116273	rs722081	T	A	0.718	-0.081	0.019	0.737	3.816	2.76E-05	1.02E-04	C14orf161
SNP_A-1941908	10	123762573	rs10788229	A	G	0.581	-0.075	0.018	0.761	3.809	2.81E-05	1.04E-04	TACC2
SNP_A-4279107	7	32100547	rs11768207	G	C	0.658	0.076	0.018	0.718	3.805	2.84E-05	1.05E-04	AK091734
SNP_A-2237883	6	114006654	rs4341037	C	G	0.67	-0.079	0.019	0.758	3.793	2.93E-05	1.07E-04	-
SNP_A-2116582	20	46471709	rs4810788	C	A	0.745	0.086	0.021	0.782	3.785	2.98E-05	1.09E-04	-
SNP_A-2313081	2	59136116	rs1024766	G	A	0.652	-0.076	0.018	0.73	3.775	3.05E-05	1.11E-04	-
SNP_A-1808597	18	43889962	rs11664553	G	T	0.843	-0.099	0.024	0.725	3.746	3.28E-05	1.18E-04	-
SNP_A-4256844	10	123761293	rs12571870	A	C	0.582	-0.074	0.018	0.745	3.74	3.32E-05	1.20E-04	TACC2
SNP_A-4280537	14	91113496	rs1704609	G	C	0.719	-0.08	0.019	0.722	3.739	3.33E-05	1.20E-04	C14orf161
SNP_A-1805190	7	122863069	rs4731112	C	G	0.676	0.077	0.019	0.73	3.732	3.39E-05	1.22E-04	CR749438, WASL, ASB15
SNP_A-2223873	10	77343635	rs11816174	A	G	0.676	0.079	0.019	0.767	3.725	3.44E-05	1.24E-04	C10orf11
SNP_A-2276112	11	127376187	rs11221126	C	A	0.543	0.072	0.017	0.705	3.725	3.45E-05	1.24E-04	-
SNP_A-1838872	5	79411480	rs2288395	G	C	0.723	0.08	0.019	0.708	3.712	3.56E-05	1.27E-04	THBS4
SNP_A-2202767	3	140326401	rs661149	G	A	0.767	0.084	0.02	0.7	3.683	3.82E-05	1.35E-04	BPESC1
SNP_A-1895268	12	94498374	rs10859907	T	C	0.865	-0.104	0.025	0.696	3.676	3.89E-05	1.37E-04	-
SNP_A-2194277	10	38131154	rs3739992	G	A	0.772	0.086	0.021	0.73	3.668	3.96E-05	1.39E-04	AK027057, ZNF25, ZNF33A, BC036038
SNP_A-1958937	5	110218300	rs13357752	A	G	0.778	0.085	0.021	0.699	3.63	4.34E-05	1.51E-04	-
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SNP_A-2271920	5	97244183	rs6859385	T	G	0.841	-0.098	0.024	0.711	3.602	4.64E-05	1.60E-04	-
SNP_A-2232006	20	46495329	rs6095120	G	C	0.781	0.088	0.022	0.734	3.57	5.02E-05	1.71E-04	-
SNP_A-1836879	5	110189680	rs12517265	C	T	0.775	0.085	0.021	0.704	3.569	5.03E-05	1.72E-04	-
SNP_A-4267149	8	109621322	rs10505124	A	C	0.602	0.073	0.018	0.71	3.566	5.07E-05	1.73E-04	KIAA0103
SNP_A-4267548	9	83119573	rs1359169	G	A	0.868	0.105	0.026	0.708	3.565	5.08E-05	1.73E-04	BC037253, FRMD3, AY137774, BC023560, AK223597, AK094281
SNP_A-2170138	12	117295164	rs17440956	G	A	0.865	-0.105	0.026	0.724	3.542	5.38E-05	1.82E-04	SDS3, BC002756, SUDS3, TAOK3
SNP_A-2050003	6	12999287	rs7750679	T	C	0.505	-0.072	0.018	0.718	3.54	5.41E-05	1.82E-04	PHACTR1, BC047159
SNP_A-2006708	2	132991575	rs2315380	T	C	0.723	-0.081	0.02	0.727	3.535	5.47E-05	1.84E-04	-
SNP_A-2233461	6	7506660	rs2757632	A	G	0.583	-0.071	0.018	0.673	3.533	5.49E-05	1.85E-04	DSP
SNP_A-4244296	9	83132937	rs1323780	C	A	0.871	0.106	0.026	0.704	3.516	5.72E-05	1.92E-04	BC037253, FRMD3, AY137774, BC023560, AK223597, AK094281
SNP_A-2312625	3	195801179	rs1706016	C	T	0.54	0.072	0.018	0.709	3.512	5.78E-05	1.93E-04	AY597813, BC034353, AY303778, BC007772, TMEM44, AK126914
SNP_A-1892007	7	32103576	rs10271037	G	T	0.654	0.073	0.018	0.662	3.511	5.79E-05	1.94E-04	AK091734
SNP_A-1880815	3	195800787	rs1706017	C	T	0.541	0.072	0.018	0.708	3.511	5.80E-05	1.94E-04	AY597813, BC034353, AY303778, BC007772, TMEM44, AK126914
SNP_A-2039539	19	17656360	rs8106359	G	C	0.516	0.074	0.018	0.751	3.498	5.97E-05	1.99E-04	-
SNP_A-2077776	1	40874545	rs4660449	G	T	0.726	0.079	0.02	0.693	3.485	6.18E-05	2.05E-04	AK094323, AK127677, RIMS3, NFYC
SNP_A-2176681	6	13002890	rs9296512	G	C	0.51	-0.071	0.018	0.706	3.481	6.23E-05	2.07E-04	PHACTR1, BC047159
SNP_A-2169005	9	16844839	rs1339550	C	T	0.902	-0.113	0.028	0.63	3.471	6.39E-05	2.11E-04	BNC2
SNP_A-4194655	4	171572150	rs7698213	G	A	0.563	0.073	0.018	0.728	3.468	6.44E-05	2.12E-04	-
SNP_A-2060462	8	125011048	rs7838453	C	A	0.682	-0.073	0.018	0.643	3.454	6.66E-05	2.19E-04	C8ORFK23
SNP_A-1914252	1	100883020	rs11578560	C	T	0.546	0.07	0.018	0.676	3.449	6.73E-05	2.21E-04	VCAM1
SNP_A-2249896	5	110225865	rs6888588	A	G	0.777	0.084	0.021	0.687	3.443	6.83E-05	2.24E-04	-

SNP_A-2190693	17	23130802	rs4795067	A	G	0.55	0.069	0.017	0.656	3.432	7.02E-05	2.29E-04	NOS2A
SNP_A-2160094	1	40847036	rs2744808	C	T	0.724	0.079	0.02	0.686	3.428	7.09E-05	2.31E-04	AK094323, AK127677, RIMS3, NFYC
SNP_A-2283289	5	23488681	rs2914263	G	A	0.911	0.121	0.031	0.662	3.422	7.19E-05	2.34E-04	-
SNP_A-2136756	7	28267685	rs6977204	G	A	0.616	-0.071	0.018	0.662	3.422	7.20E-05	2.34E-04	CREB5
SNP_A-1818349	11	65339642	rs3903072	T	G	0.516	-0.07	0.018	0.688	3.402	7.55E-05	2.44E-04	FLJ30934, MUS81, BC040981, EFEMP2
SNP_A-1783528	7	32084966	rs1450870	C	T	0.58	0.07	0.018	0.659	3.392	7.74E-05	2.49E-04	AK091734
SNP_A-2234780	17	23121258	rs9797244	T	C	0.616	0.072	0.018	0.672	3.369	8.18E-05	2.62E-04	NOS2A
SNP_A-1906570	5	154598503	rs6580149	G	A	0.808	-0.086	0.022	0.632	3.355	8.47E-05	2.69E-04	-

*- adjusted p-values according to the genomic control method (Devlin B, Roeder K. Genomic control for association studies. *Biometrics.* 1999;55:997-1004

SUPPLEMENTAL FIGURES

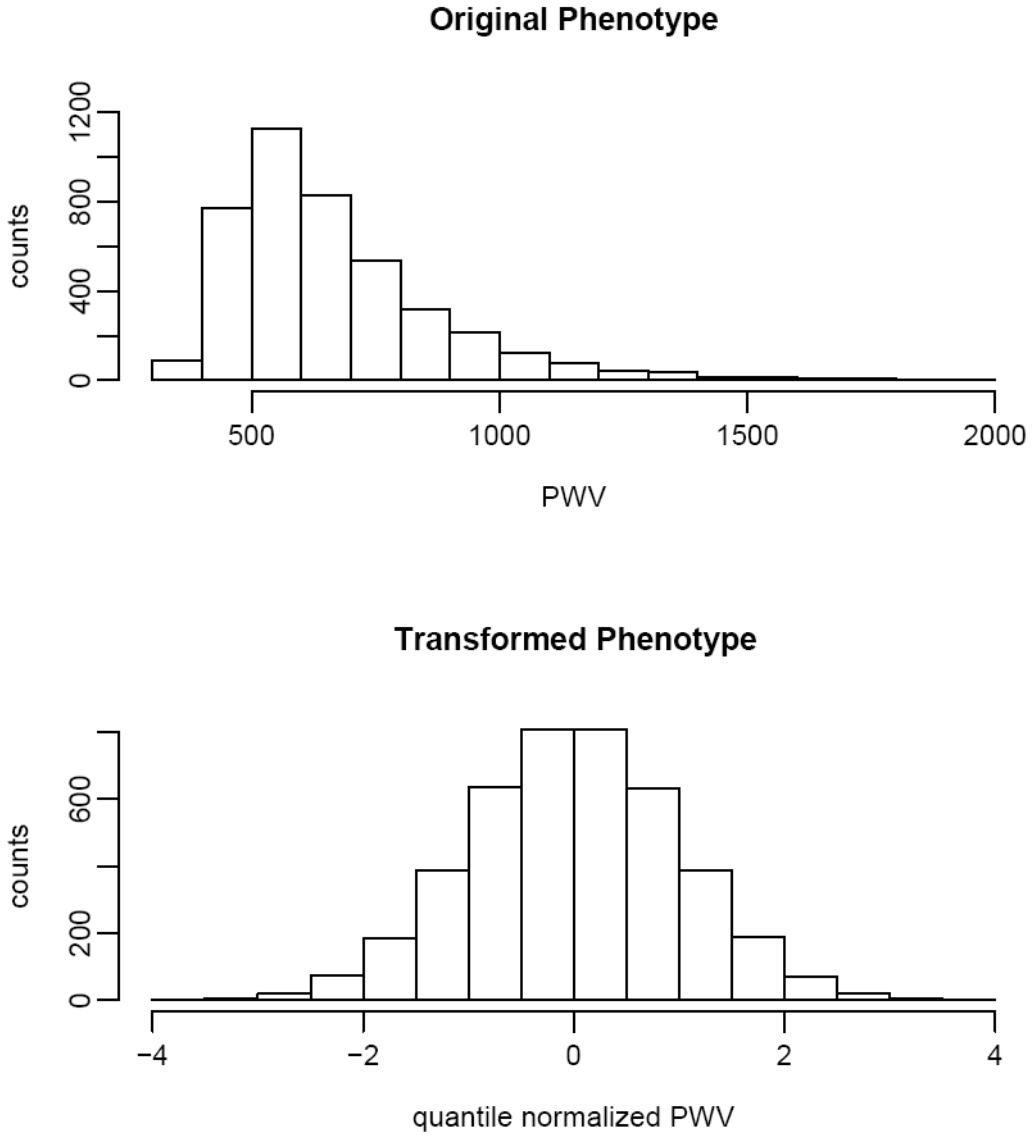

Figure S1

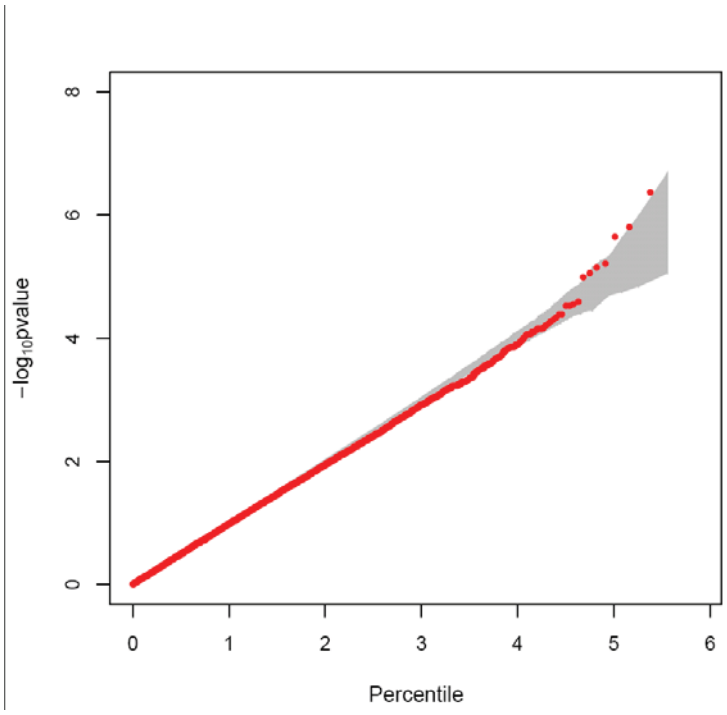

Figure S2

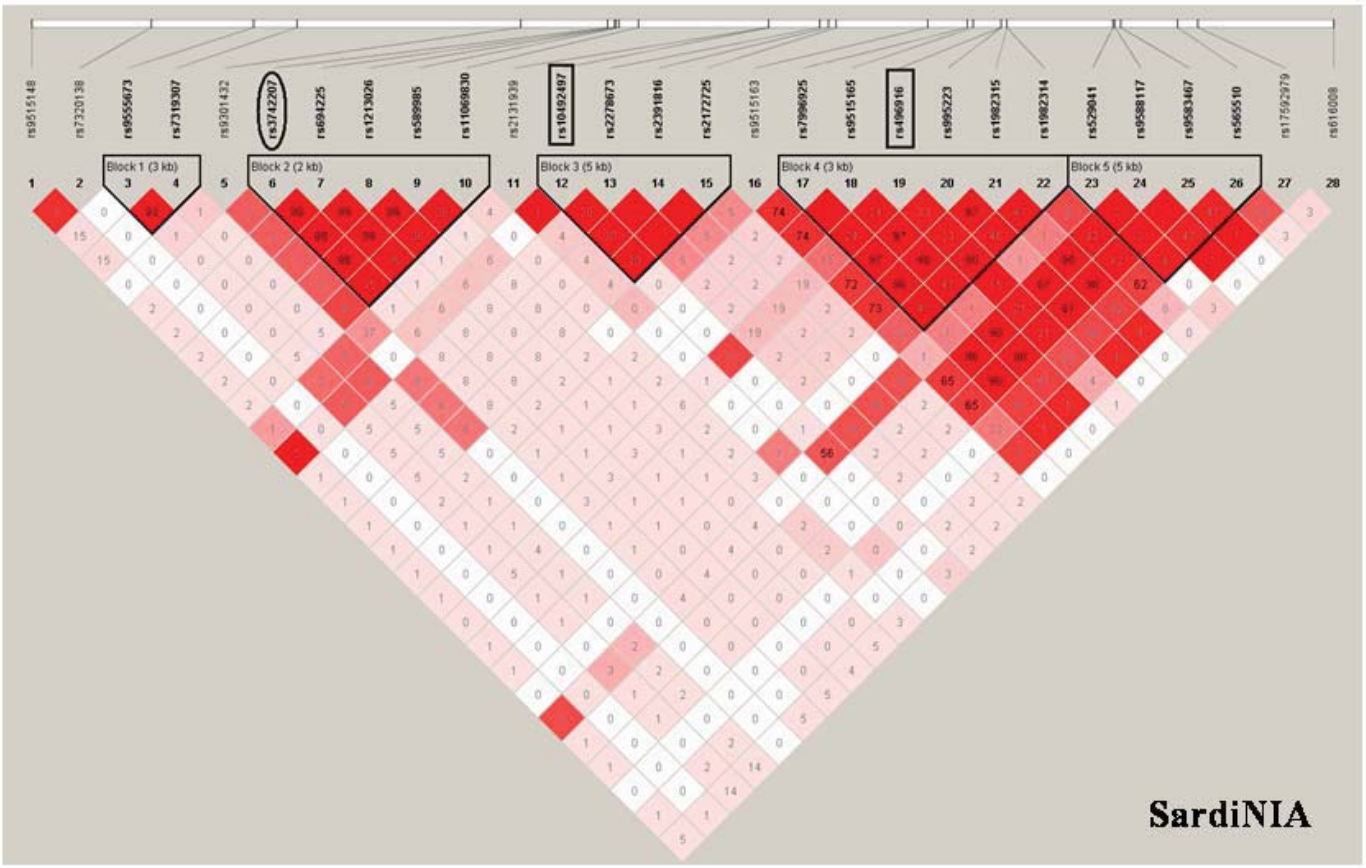

Figure S3

Figure Legends

Figure S1: Frequency distribution of PWV values before and after inverse normal transformation. The raw values have a skewed distribution. The transformed values appear normally distributed.

Figure S2: Quantile-quantile plot of SNPs associated with PWV in the SardiNIA study after adjusting the p-values according to the genomic control method[15]. Red symbols represent all tested SNPs (N= 362,129) in the GWA scan. The gray area corresponds to the 90% confidence region from a null distribution of p-values (generated from 100 simulations).

Figure S3. High resolution linkage disequilibrium plot and LD blocks in the Sardinian (top panel) and Old Order Amish (bottom panel) populations around SNP rs3742207 (highlighted with a circle) from Haploview[16]. LD coloring scheme is the standard D'/LOD scheme, while numbers shown within squares are r^2 values. Genotyped SNPs in the region slightly differ between the two cohorts, but clearly define the length of the second haplotype block, delimited by SNPs rs3742207 and rs11069830, and of identical length in the HapMap CEU population. Squared SNPs are those analyzed by the Framingham study. No other SNP analyzed by Framingham falls in this region.

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